

Asymmetric dimethylarginine is transported by the mitochondrial carrier SLC25A2

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Abstract Asymmetric dimethyl L-arginine (ADMA) is generated within cells and in mitochondria when proteins with dimethylated arginine residues are degraded. The aim of this study was to identify the carrier protein(s) that transport ADMA across the inner mitochondrial membrane. It was found that the recombinant, purified mitochondrial solute carrier SLC25A2 when reconstituted into liposomes efficiently transports ADMA in addition to its known substrates arginine, lysine, and ornithine and in contrast to the other known mitochondrial amino acid transporters SLC25A12, SLC25A13, SLC25A15, SLC25A18, SLC25A22, and SLC25A29. The widely expressed SLC25A2 transported ADMA across the liposomal membrane in both directions by both unidirectional transport and exchange against arginine or lysine. The SLC25A2-mediated ADMA transport followed first-order kinetics, was nearly as fast as the transport of the best SLC25A2 substrates known so far, and was highly specific as symmetric dimethylarginine (SDMA) was not transported at all. Furthermore, ADMA inhibited SLC25A2 activity with an inhibition constant of 0.38 ± 0.04 mM, whereas SDMA inhibited it poorly. We propose that a major function of SLC25A2 is to export ADMA from mitochondria missing the mitochondrial ADMA-metabolizing enzyme AGXT2. There is evidence that ADMA can also be imported into

mitochondria, e.g., in kidney proximal tubulus cells, to be metabolized by AGXT2. SLC25A2 may also mediate this transport function.

Keywords ADMA · Membrane transport · Mitochondrial carrier · Mitochondrial transporter · Mitochondria · SLC25A2

Abbreviations

ADMA Asymmetric dimethylarginine
SDMA Symmetric dimethylarginine
SLC25 Solute carrier family 25
HEPES *N*-(2-Hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)

Introduction

Asymmetric dimethyl L-arginine (ADMA) is an independent risk factor for cardiovascular disease. There is increasing evidence indicating that ADMA causes cardiovascular dysfunction and disease, most likely through its competition with L-arginine at the substrate-binding site of endothelial nitric oxide synthase (eNOS) and consequently inhibition of nitric oxide (NO) synthesis (Leiper and Nandi 2011; Caplin and Leiper 2012; Böger et al. 2009; Anderssohn et al. 2010). Increased ADMA is also associated with an increased production of reactive oxygen species (ROS) (Wilcox 2012). NO produced by eNOS serves to dilate blood vessels and to protect them from atherosclerosis (Förstermann and Münzel 2006). ROS on the other hand have detrimental effects on the cardiovascular system. In addition, they react very rapidly with NO to produce reactive nitrogen–oxygen species such as peroxynitrite thus transforming a vasoprotector into vasotoxic agents.

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In spite of the well-known inhibitory action of ADMA on eNOS, little is known about the intracellular distribution of this arginine derivative in endothelial cells and other cell types. ADMA is generated within cells when proteins with dimethylated arginine residues are degraded. Protein degradation occurs not only in the cytosol, but also in mitochondria (Koppen and Langer 2007), suggesting that ADMA is generated within and needs to be eliminated from these organelles. Endothelial cells can dispose of ADMA by degradation through the cytosolic enzyme dimethyl arginine dimethylamino hydrolase (DDAH) (Birdsey et al. 2000). Recent work has shown that cellular efflux mediated by system y^+L amino acid transporters (y^+LATs) may even be more important to lower intracellular ADMA concentrations (Closs et al. 2012). However, neither elimination pathways would lower mitochondrial ADMA concentrations, unless ADMA was exported from these organelles into the cytoplasm. In addition to DDAH, a second ADMA-degrading enzyme has been identified in proximal tubular cells of the kidney, alanine:glyoxylate amino-transferase (AGXT2). AGXT2 localizes in mitochondria and metabolizes significant amounts of circulating ADMA (Rodionov et al. 2010). The effectiveness of AGXT2 to degrade extracellular ADMA must thus depend on plasma membrane and mitochondrial transporters. Accordingly, there are two conceivable scenarios where mitochondrial ADMA transport may be important: (1) in cells missing mitochondrial AGXT2 (most non-kidney cells) to eliminate ADMA generated within mitochondria and (2) in kidney proximal tubular cells to transport ADMA generated in the cytosol or taken up from the circulation to the ADMA-degrading enzyme AGXT2 located within mitochondria.

So far, the carrier proteins that transport ADMA across the inner mitochondrial membranes had not been known. We therefore asked what transporters are responsible for mitochondrial ADMA in- and export. Because ADMA is an arginine derivative and a cationic amino acid, we examined if members of the mitochondrial carrier family that accept cationic or other amino acids as a substrate (for reviews see Palmieri 2013, 2014), also translocate ADMA across the mitochondrial membrane. In this study, we provide evidence that the mitochondrial carrier family member SLC25A2 is a transporter of ADMA. SLC25A2 overexpressed in *Escherichia coli* and reconstituted in phospholipid vesicles transported ADMA across the liposomal membrane with high specificity and high affinity. In contrast, neither the mitochondrial carriers SLC25A15 and SLC25A29, that also transport cationic amino acids, nor any of the other known mitochondrial amino acid carriers (SLC25A12, SLC25A13, SLC25A18, and SLC25A22) did accept ADMA as a substrate.

Experimental procedures

Materials

Radioactive compounds were purchased from Perkin Elmer, USA, Scopus Research BV, The Netherlands, and Hartmann Analytic, Germany. N^G,N^G -dimethyl-L-arginine dihydrochloride was purchased from Santa Cruz Biotechnology, USA. All other compounds were obtained from Sigma Aldrich, USA.

Construction of expression plasmids

The coding sequences for SLC25A2 (NM_031947), SLC25A12 (NM_003705), SLC25A13 (NM_014251), SLC25A15 (NM_014252), SLC25A18 (NM_031481), SLC25A22 (NM_024698), and SLC25A29 (NM_001039355) were amplified as perviously described (Fiermonte et al. 2002, 2003; Lasorsa et al. 2003; Monné et al. 2012; Porcelli et al. 2014). The amplified products were cloned into the pRUN (SLC25A2, SLC25A15, SLC25A18, SLC25A22, and SLC25A29), the pMW7 (SLC25A12), and pET-15b (SLC25A13) expression vectors and transformed into *E. coli* DH5 α cells. Transformants were selected on ampicillin (100 μ g/ml) and screened by direct colony PCR. The sequences of the inserts were verified by DNA sequencing.

Bacterial expression and purification of recombinant proteins

SLC25A2, SLC25A12, SLC25A13, SLC25A15, SLC25A18, SLC25A22, and SLC25A29 were overexpressed as inclusion bodies in the cytosol of *E. coli* as previously described (Fiermonte et al. 2001, 2009; Palmieri et al. 2006; Marobbio et al. 2006); host cells were *E. coli* Rosetta-gami B[DE3] for SLC25A29 and *E. coli* CO214[DE3] for all the other proteins. Control cultures with empty vector were processed in parallel. Inclusion bodies were purified on a sucrose density gradient and washed at 4 °C first with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0), then twice with a buffer containing Triton X-114 (3 %, w/v), 1 mM EDTA, and 10 mM HEPES, pH 7.2, and lastly twice with TE buffer (Marobbio et al. 2003; Palmieri et al. 2008). Finally, proteins were solubilized in 1.7–1.9 sarkosyl (w/v) and small residues were removed by centrifugation (20,800 \times g for 10 min at 4 °C).

Reconstitution into liposomes

The recombinant proteins in sarkosyl were reconstituted into liposomes by cyclic removal of the detergent with a hydrophobic column of Amberlite beads (Fluka) as described previously (Palmieri et al. 1995, 2009; Agrimi

et al. 2012) with some modifications. The initial mixture used for reconstitution contained solubilized proteins (2–5 μg), 1 % Triton X114, 1 % egg yolk phospholipids in the form of sonicated liposomes, 10 mM substrate, 20 mM HEPES (pH 7.2), 0.8 mg of cardiolipin, and water to a final volume of 700 μl . After vortexing, this mixture was recycled 13 times through an Amberlite column pre-equilibrated with a buffer containing 10 mM HEPES and 50 mM NaCl (pH 7.2), and the substrate at the same concentration as in the starting mixture.

Transport measurements

The external substrate present in the reconstitution mixture (see section “[Reconstitution into liposomes](#)”) was removed from proteoliposomes on Sephadex G-75 columns pre-equilibrated with 50 mM NaCl and 10 mM HEPES at pH 7.2. Transport was started at 25 °C by adding radioactive substrate ($[^3\text{H}]$ arginine, $[^3\text{H}]$ lysine, or $[^3\text{H}]$ ornithine for SLC25A2; $[^3\text{H}]$ ornithine or $[^3\text{H}]$ lysine for SLC25A15; $[^{14}\text{C}]$ aspartate or $[^{14}\text{C}]$ glutamate for SLC25A12 and SLC25A13; $[^{14}\text{C}]$ glutamate for SLC25A18 and SLC25A22; and $[^3\text{H}]$ arginine or $[^3\text{H}]$ lysine for SLC25A29) to preloaded or unloaded proteoliposomes. In all cases, transport was terminated by addition of 30 mM pyridoxal 5'-phosphate and 15 mM bathophenanthroline which in combination and at high concentrations inhibit the activity of several mitochondrial carriers rapidly and completely (Palmieri et al. 1997a, 2006; Floyd et al. 2007; Wibom et al. 2009; Castegna et al. 2010). In control samples, the inhibitors were added at the beginning together with the external substrate. Finally, the external substrate was removed by Sephadex G75 columns, and radioactivity in the liposomes was measured. The experimental values were corrected by subtracting control values. The initial transport rate was calculated from the radioactivity taken up by the proteoliposomes in the initial linear range of substrate uptake.

Other methods

Proteins were analyzed by SDS-PAGE and stained with Coomassie Blue dye. The amount of pure recombinant proteins was estimated by laser densitometry of stained samples, using carbonic anhydrase as protein standard (Marobio et al. 2008; Di Noia et al. 2014). The identity of purified proteins was assessed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry of trypsin digests of the corresponding band excised from a Coomassie-stained gel (Palmieri et al. 2001; Hoyos et al. 2003). To assay the protein incorporated into liposomes, the vesicles were passed through a Sephadex G-75 column, centrifuged at $300,000\times g$ for 30 min, and delipidated with

organic solvents as described in Capobianco et al. (1996). Then, the SDS-solubilized protein was determined by comparison with carbonic anhydrase in SDS gels. The share of incorporated protein was about 20–25 % of the protein added to the reconstitution mixture.

Results

ADMA is transported by SLC25A2

Given that ADMA is an analog of L-arginine, the three mitochondrial carriers known to transport arginine, i.e., SLC25A2, SLC25A15, and SLC25A29 (Camacho et al. 1999; Fiermonte et al. 2003; Porcelli et al. 2014; Monné et al. 2015), were investigated for their ability to transport ADMA upon expression in *E. coli*, purification, and incorporation into liposomes. As radioactive ADMA and SDMA would only have been available through custom synthesis, a labeled compound known to be transported by the investigated carrier was added to reconstituted liposomes preloaded with 10 mM ADMA, SDMA, or (as positive control) the same substrate used externally in these experiments (Fig. 1). In the case of SLC25A2, the hetero-exchanges $[^3\text{H}]$ arginine/ADMA, $[^3\text{H}]$ lysine/ADMA, or $[^3\text{H}]$ ornithine/ADMA were high and comparable with the homo-exchanges $[^3\text{H}]$ arginine/arginine, $[^3\text{H}]$ lysine/lysine, or $[^3\text{H}]$ ornithine/ornithine, respectively (Fig. 1a). By contrast, despite the long incubation period (i.e., 120 min), a very low uptake of labeled substrate was observed in liposomes reconstituted with either SLC25A15 or SLC25A29 and containing ADMA internally (Fig. 1b, c). The amount of radioactivity taken up by these liposomes was virtually the same as that entering by uniport in the absence of any internal substrate. Furthermore, $[^3\text{H}]$ arginine, $[^3\text{H}]$ lysine, or $[^3\text{H}]$ ornithine uptake via SLC25A2; $[^3\text{H}]$ ornithine or $[^3\text{H}]$ lysine uptake via SLC25A15; and $[^3\text{H}]$ arginine or $[^3\text{H}]$ lysine uptake via SLC25A29 into liposomes preloaded with internal SDMA were virtually the same as that into liposomes without internal substrate. This lack of trans-stimulation indicates that SDMA is not transported by any of these carriers. Next, the ability of the other mitochondrial amino acid carriers, i.e., the two isoforms of the aspartate-glutamate carrier (SLC25A12 and SLC25A13) and of the glutamate carrier (SLC25A18 and SLC25A22), to transport ADMA or SDMA was tested. Using liposomes reconstituted with SLC25A12 or SLC25A13 (that catalyze only an exchange of substrates) and preloaded with internal ADMA or SDMA, no uptake of $[^{14}\text{C}]$ aspartate or $[^{14}\text{C}]$ glutamate was observed after 60-min incubation (data not shown). Likewise, using SLC25A18- and SLC25A22-reconstituted liposomes (that catalyze both uniport and exchange), the uptake of $[^{14}\text{C}]$ glutamate against internal

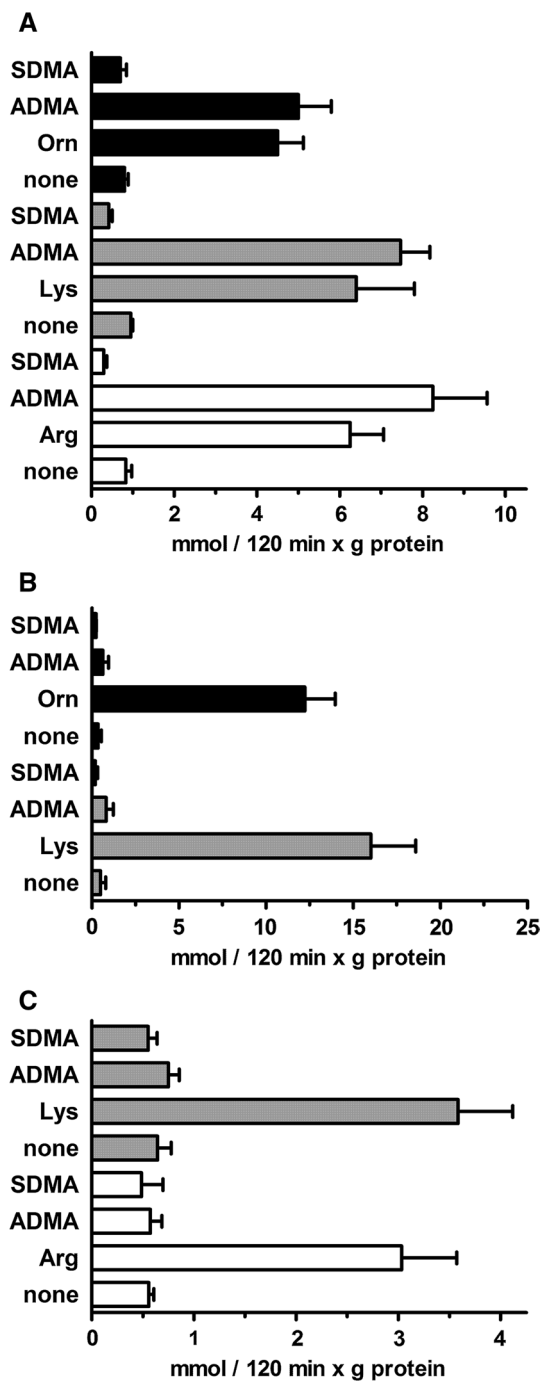


Fig. 1 Ability of mitochondrial carriers for cationic amino acids to transport ADMA. Liposomes were reconstituted with recombinant SLC25A2 (a), SLC25A15 (b), or SLC25A29 (c) and preloaded internally with the indicated substrates (concentration, 10 mM). Transport was started by adding 0.7 mM [^3H]arginine, [^3H]lysine, or [^3H]ornithine (SLC25A2), [^3H]lysine or [^3H]ornithine (SLC25A15) or [^3H]arginine or [^3H]lysine (SLC25A29). Incubation time was 120 min. Data are means \pm SD of at least three independent experiments. *White, gray, and black columns* represent the samples to which [^3H]arginine, [^3H]lysine, or [^3H]ornithine, respectively, was added

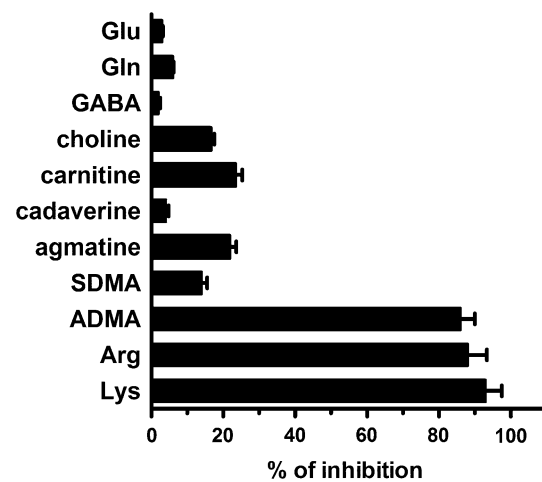


Fig. 2 Inhibition of SLC25A2 activity by ADMA. Liposomes were reconstituted with SLC25A2 and preloaded internally with 10 mM lysine. Transport was initiated by adding 0.35 mM [^3H]lysine with and without the indicated compounds (concentration, 4 mM). The reaction time was 2 min. The data expressed as percentage of inhibition are means \pm SD of at least three independent experiments

ADMA or SDMA, after 60-min incubation, was not significantly different from that measured in the absence of internal substrate (data not shown). These results indicate that among the mitochondrial carriers investigated, SLC25A2 is the only one capable of transporting ADMA, whereas SDMA is not transported by any of them.

Inhibition of SLC25A2 activity by ADMA

To further evaluate whether ADMA is a substrate of SLC25A2, we tested its ability to inhibit the initial rate of the [^3H]lysine/lysine exchange in liposomes reconstituted with recombinant SLC25A2. As shown in Fig. 2, 4 mM ADMA markedly decreased the uptake of 0.35 mM radioactive [^3H]lysine into the liposomes. Likewise, the [^3H]lysine/lysine exchange activity was inhibited by known substrates (lysine and arginine) of SLC25A2 to the same extent. By contrast, 4 mM SDMA had little effect on the uptake of radioactive [^3H]lysine into the liposomes. Furthermore, other externally added compounds, structurally related to arginine or lysine (agmatine, cadaverine, carnitine, choline, glutamine, and GABA), had a little or no significant effect on the lysine/lysine exchange catalyzed by reconstituted SLC25A2.

Kinetic characteristics of SLC25A2-catalyzed ADMA transport

The time courses of 1 mM [^3H]arginine and 1 mM [^3H]lysine uptake into liposomes reconstituted with SLC25A2

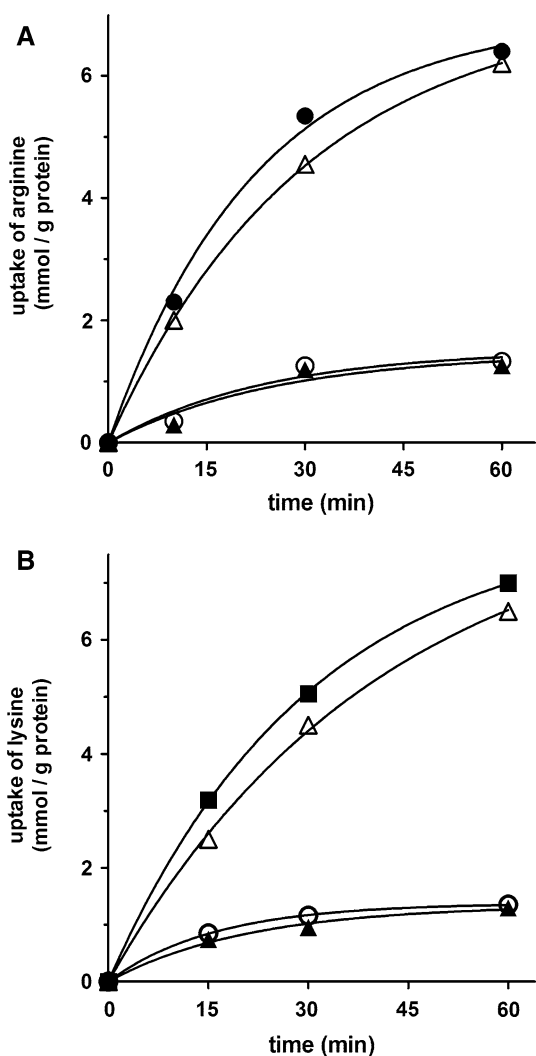


Fig. 3 Time courses of [^3H]arginine or [^3H]lysine uptake by SLC25A2-reconstituted liposomes. Proteoliposomes were preloaded internally with 10 mM arginine (filled circles) or lysine (filled squares), 10 mM ADMA (open triangles), 10 mM SDMA (filled triangles), or 10 mM NaCl and no substrate (open circles). Transport was initiated by adding 1 mM [^3H]arginine in **a** or [^3H]lysine in **b** and terminated at the indicated times. Similar results were obtained in three different experiments

were measured either as exchange (with 10 mM ADMA inside the proteoliposomes) or as uniport (in the absence of internal substrate) (Fig. 3a and b, respectively). In both modes, isotopic equilibrium was approached exponentially in accord with transport by first-order kinetics. The initial rates of the [^3H]arginine/arginine and [^3H]arginine/ADMA exchanges, deduced from the time courses (Palmieri et al. 1995), were 0.29 and 0.24 mmol/min \times g of protein, respectively; those of the [^3H]lysine/lysine and [^3H]lysine/ADMA exchanges were 0.26 and 0.21 mmol/min \times g of protein, respectively. The initial rates of the [^3H]arginine and [^3H]lysine uniport were 0.05 and 0.08 mmol/min \times g

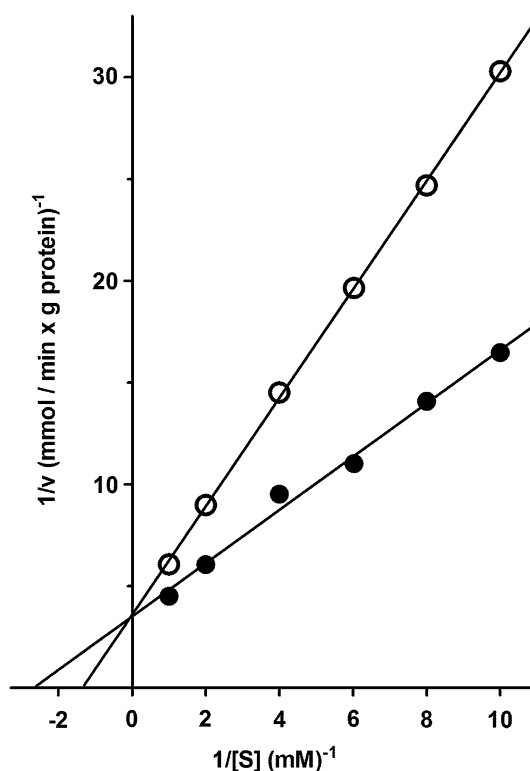


Fig. 4 Competitive inhibition of the [^3H]lysine/lysine exchange by ADMA in liposomes reconstituted with SLC25A2. [^3H]lysine was added at the concentrations of 0.1, 0.125, 0.166, 0.25, 0.5, and 1.0 mM to proteoliposomes containing 10 mM intravesicular lysine. The radioactive substrate was added in the presence (open circles) or absence (filled circles) of 0.25 mM ADMA. The incubation time was 2 min. The data from one representative experiment are reported

of protein, respectively. Therefore, SLC25A2 catalyzes both unidirectional transport and exchange of substrates, though the rate of the former is about one-fourth of the latter. Notably, the activities and extents of the [^3H]arginine/ADMA and [^3H]lysine/ADMA exchanges were similar to those of the arginine and lysine homoexchanges. Furthermore, the uptake of [^3H]arginine or [^3H]lysine into SDMA-containing liposomes was almost identical to that measured in the absence of any substrate inside the proteoliposomes (Fig. 3a, b), confirming that SDMA is not transported by SLC25A2.

The kinetic constants of the reconstituted [^3H]lysine/ADMA exchange were determined by measuring the initial transport rate at various external [^3H]lysine concentrations, in the presence of a constant saturating internal concentration of 10 mM ADMA. For the [^3H]lysine/ADMA exchange at 25 $^{\circ}\text{C}$, the average half-saturation constant (K_m) was 0.37 ± 0.04 mM and the average specific activity (V_{max}) was 0.31 ± 0.05 mmol/min/g protein in seven experiments (data not shown). Furthermore, ADMA inhibited the [^3H]lysine/lysine exchange competitively by increasing

the apparent K_m without changing the V_{max} of [^3H]lysine uptake (Fig. 4). The average inhibition constant (K_i) of ADMA was 0.38 ± 0.04 mM in five experiments.

ADMA-induced efflux of [^3H]arginine or [^3H]lysine from SLC25A2-reconstituted liposomes

In another set of experiments, the ability of ADMA to induce the efflux of 1 mM [^3H]arginine or [^3H]lysine from liposomes reconstituted with recombinant SLC25A2 was investigated. First, unloaded proteoliposomes were incubated with 1 mM [^3H]arginine or [^3H]lysine for 90 min, when the uptake of labeled substrate had almost reached equilibrium (Fig. 5). Then, 20 mM unlabeled ADMA was added externally. This caused an extensive efflux of radiolabeled arginine and lysine, respectively. This shows that the [^3H]arginine or [^3H]lysine taken up by uniport into SLC25A2-reconstituted liposomes is released by exchange for externally added ADMA. By contrast, the addition of 20 mM SDMA (Fig. 5) or agmatine (not shown) had virtually no effect on the intraliposomal lysine or arginine content. Therefore, ADMA is transported by reconstituted SLC25A2 not only when present inside liposomes but also when added externally, whereas SDMA and agmatine are not.

Discussion

With the work presented here, we have identified SLC25A2 as a mitochondrial transporter for ADMA. Our transport measurements demonstrate that ADMA is transported efficiently by SLC25A2. In contrast, none of the other known mitochondrial amino acid transporters (SLC25A12, SLC25A13, SLC25A15, SLC25A18, SLC25A22, and SLC25A29) transported ADMA. Notably, given that all the physiological substrates of SLC25A2 except for ADMA are also transported by SLC25A15 and/or SLC25A29, a major distinct function of SLC25A2 is to translocate ADMA across the mitochondrial membrane.

The SLC25A2-mediated transport of ADMA followed first-order kinetics, displayed an affinity for the carrier similar to that exhibited by the best substrates of SLC25A2, i.e., lysine and arginine, and was highly specific as SDMA was not transported at all. In agreement with being a good substrate, ADMA strongly inhibited SLC25A2 activity similar to lysine and arginine. SDMA, as well as other related compounds, though not transported, displayed some inhibition on the activity of SLC25A2. This has also been observed with other carriers and other compounds (Palmieri et al. 1997b; Fiermonte et al. 2003; Marobbio et al. 2008; Porcelli et al. 2014). This behavior can be explained by the ability

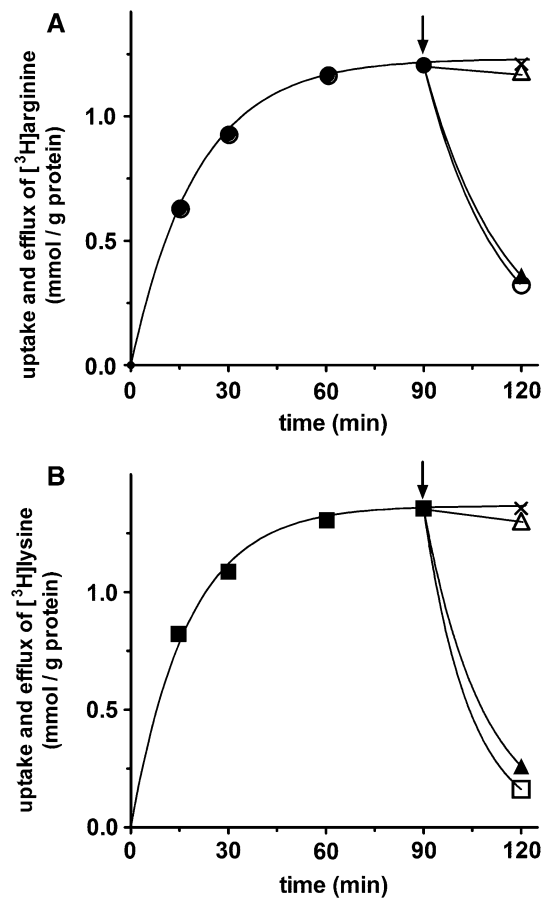


Fig. 5 Uptake of [^3H]arginine or [^3H]lysine into unloaded proteoliposomes and their efflux after addition of unlabeled substrate. 1 mM [^3H]arginine (filled circles in a) or 1 mM [^3H]lysine (filled squares in b) was added to liposomes reconstituted with SLC25A2 and containing 10 mM NaCl and no substrate. The arrows indicate the addition of extravesicular 20 mM arginine (open circles in a) or lysine (open squares in b), 20 mM ADMA (filled triangles), 10 mM SDMA (open triangles), each in a buffer consisting of 50 mM NaCl and 10 mM HEPES at pH 7.2, or buffer alone (times symbol). The data from one representative experiment are reported

of SDMA (and other inhibiting compounds) to partially interfere with the binding of transportable substrates to the carrier and its inability to trigger the conformational changes required for carrying on the translocation process (Palmieri and Pierri 2010a, b). Furthermore, it is noteworthy that ADMA was transported by SLC25A2 across the liposomal membrane in both directions unidirectionally or, with a higher rate, by exchange with other substrates. Because ADMA carries a positive charge, its unidirectional uptake into respiring mitochondria should be favored by the electrical component (negative inside) of the proton motive force generated by electron transport (Mitchell 1961). Consequently, its efflux should be hampered by the mitochondrial membrane potential. It could be argued, therefore, that, under physiological

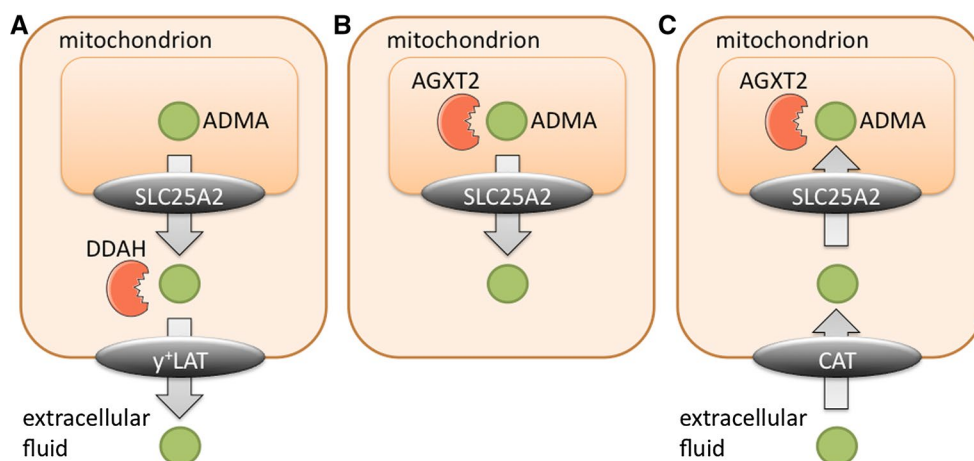


Fig. 6 Proposed roles of SLC25A2 in ADMA metabolism. **a, b** The fate of ADMA generated within mitochondria by degradation of proteins containing asymmetrically dimethylated arginine residues: **a** in cells missing the mitochondrial ADMA-degrading enzyme AGXT2, SLC25A2 represents the exclusive elimination path exporting ADMA to the cytoplasm, where ADMA may be degraded by cytoplasmic DDAH or be exported by plasma membrane transporters (γ^+ LAT) into the extracellular fluid; and **b** in cells expressing AGXT2, ADMA may be degraded by AGXT2

or be exported by SLC25A2 from the mitochondria. This SLC25A2-mediated ADMA export may be less important or even unfavorable in case the cytoplasmic routes are missing. **c** The possibility that ADMA is imported into mitochondria by SLC25A2: in cells containing mitochondrial AGXT2 and low or missing DDAH activity and γ^+ LAT expression, SLC25A2 may serve to import ADMA, generated in the cytoplasm or taken up from the extracellular fluid by plasma membrane transporters (CAT), into mitochondria where it is degraded by AGXT2

conditions, the unidirectional efflux from the mitochondria is unlikely to occur. However, on the basis of the data of this paper, ADMA can be exported by SLC25A2 in exchange with the cationic amino acid lysine or arginine by an electroneutral mode of transport. In separate experiments, we found that mono-methylarginine was transported efficiently by SLC25A2 and SLC25A29 (but not by SLC25A15) in exchange for arginine and lysine (data not shown). The inhibition constant (K_i) of mono-methylarginine for SLC25A2 (0.35 ± 0.03 mM) was very similar to that of ADMA.

ADMA is generated during proteolysis of proteins containing asymmetrically dimethylated arginine residues. It can then be either enzymatically metabolized or exported into the blood to be taken up by other cells for metabolism. Part of plasma ADMA is also excreted by the kidney (Teerlink 2005). Two kinds of ADMA-metabolizing enzymes are known: the cytosolic DDAH (two isoforms) and the mitochondrial AGXT2. Mitochondrial ADMA transport could therefore have two functions (Fig. 6): (1) export of ADMA generated by mitochondrial proteolysis into the cytoplasm for cytoplasmic degradation by DDAH or for export out of the cell by plasma membrane transporters and (2) uptake of ADMA into mitochondria to enable cytoplasmic ADMA to be degraded by mitochondrial AGXT2.

1. Although type 1 protein arginine methyl transferases (PRMT), the enzymes mediating asymmetric arginine residue di-methylation in mammalian cells (Tang et al. 2000), do not seem to localize to mitochondria, argi-

nine methylated proteins may be imported into mitochondria. For example, mitochondrial localization of the BCL-2 antagonist of cell death (BAD) has been shown to depend on methylation by type 1 PRMT1 (Sakamaki et al. 2011), giving evidence for the presence of proteins with asymmetrically methylated arginine residues in mammalian mitochondria (Hockenbery et al. 1990). In addition, several proteins with these modifications have been detected in mitochondria of *Trypanosoma brucei* (Fisk et al. 2013). Degradation of such proteins thus produces ADMA within mitochondria that need to be exported, especially in the absence of a mitochondrial ADMA-metabolizing enzyme. As AGXT2 expression seems restricted to the kidney (Suhre et al. 2011; Pagliarini et al. 2008), mitochondrial ADMA export should be the important elimination route in mitochondria of most cell types. SLC25A2 is widely expressed (Fiermonte et al. 1998) and is thus likely to fulfill this function. In accordance with this assumption, we found SLC25A2 mRNA expressed in all eight human cell lines investigated (NT2 teratocarcinoma, CaCo-2 colon adenocarcinoma, DLD-1 colorectal adenocarcinoma, HuH-7 hepatocarcinoma, EA.hy926 endothelial cells, A-673 rhabdomyosarcoma, U-373 MG glioblastoma, and A549 lung carcinoma), while AGXT2 mRNA was not detectable (data not shown).

2. Several findings indicate that the mitochondrial AGXT2 metabolizes also exogenous ADMA and consequently that ADMA is transported into mitochondria

for that purpose: viral overexpression of AGXT2 in the liver leads to lowering of ADMA plasma concentrations (Rodionov et al. 2010), while deletion of a functional AGXT2 gene in mice leads to increase in plasma ADMA levels (Caplin et al. 2012). In addition, AGXT2 overexpressed in Cos7 and endothelial cells is able to degrade exogenous ADMA (Rodionov et al. 2010). However, human SNPs shown to decrease enzymatic activity of AGXT2 are not associated with elevated ADMA plasma concentrations (Kittel et al. 2014). One explanation for this discrepancy could be differences in SLC25A2 expression in the different settings, i.e., mice completely missing a functional AGXT2 may have a higher SLC25A2 expression (e.g., through compensatory upregulation) in the kidney than humans carrying mutations that decrease AGXT2 activity. This would result in ADMA efflux into the blood as observed in the mouse model, but rather mitochondrial ADMA accumulation in human kidneys (not investigated so far). In contrast to the kidney, SLC25A2 is highly expressed in liver (Fiermonte et al. 2003). AGXT2 overexpressed in this organ can therefore be expected to be efficiently supplied with substrate by SLC25A2.

SLC25A2 mRNA expression in the kidney is rather low and to date its intra-organ distribution has not been elucidated. It is therefore not clear if SLC25A2 and AGXT2 are expressed within the same cells. In situ hybridization studies localize AGXT2 mRNA exclusively in tubular epithelial cells in the Loop of Henle (Suhre et al. 2011), where neither DDAH nor an efflux transporter is found. If expressed in these cells, the main role of SLC25A2 would be substrate delivery to AGXT2. In contrast, ADMA efflux from mitochondria would rather be disadvantageous because cytoplasmic elimination routes are missing (Fig. 6b). The ADMA efflux transporter y^+ LAT1 is expressed in the proximal tubule with the highest expression near the Bowman capsule (S1 segment) (Bauch et al. 2003); y^+ LAT2 mRNA has also been detected in the kidney, but its intra-organ distribution is not known (Bröer et al. 2000). DDAH isoforms have been detected in the proximal tubule (predominantly in the S3 segment) as well as in the distal tubule and parts of the collecting duct (Onozato et al. 2008). Expression of plasma membrane exporters, cytoplasmic, and mitochondrial metabolizing enzymes for ADMA seems thus rather complementary. Expression studies localizing transporters and metabolizing enzymes in the kidney on the cellular level are needed to further elucidate the role of SLC25A2 in ADMA metabolism.

In conclusion, our identification of SLC25A2 as mitochondrial ADMA transporter together with the available expression data suggests that SLC25A2 serves as mitochondrial elimination route for ADMA in cells missing

the mitochondrial metabolizing enzyme AGXT2. If it also delivers substrate to AGXT2 in the kidney needs further investigation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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